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Certain women appear to have an increased risk of breast cancer. As compared with the general population, the first degree female relatives of breast cancer patients have been reported to have a 2- to 3-fold increase in breast cancer risk, which could be due to shared genetic and/or environmental components, and suggest a heterogeneity of population at risk. We hypothesize that persons prone for genetic instability at certain breast cancer associated gene locus are more prone to develop this cancer. We developed a novel 'Comet-FISH' technique to identify people who show genetic instability at these loci, and hence may be prone for developing breast cancer. By combining Comet assay and FISH techniques we can, not only identify cells with genetic instability, but also identify regions within the genome that are preferentially prone for instability. We applied this novel technique to look at genetic instability in predefined loci in breast cancer and normal cell lines and few normal persons. Breast cancer cell lines showed genetic instability at the HER-2 and p53 loci when examined by this Comet-FISH technique. Whereas, normal cell line and normal persons did not show genetic instability at these loci. Other genetic loci, c-myc, Rb, cyclin D and ZNF217, did not show instability. Further studies are necessary in a larger population over extended period of time, to evaluate the usefulness of this technique, for detection of persons at risk for breast cancer development.

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CONTENTS

INTRODUCTION

Certain women appear to have an increased risk of breast cancer. As compared with the general population, the first degree female relatives of breast cancer patients have been reported to have a 2- to 3-fold increase in breast cancer risk, which could be due to shared genetic and/or environmental components, and suggest a heterogeneity of population at risk(1). To date, no biomarker has been available which could be used to identify individuals at high risk(2).

The major emphasis of this study is the development and validation of a novel Comet-FISH technology that would combine the simplicity, accuracy and specificity of these 2 single cell assays. This assay would be used specifically to detect genetic instability at certain breast cancer associated genetic loci. Since the carcinogenic process generally is associated with genetic instability, we hypothesised that individuals who are prone for instability at certain breast cancer associated genetic loci will be at more risk for breast cancer development. Using this Comet-FISH technology(3, 4), we tested for genetic instability at the following breast cancer associated genetic loci:

- HER-2/neu, also known as c-erbB2 or HER-2 (17q11.2-q12), is a gene that has been shown to play a key role in the regulation of cell growth. The gene codes for a 185 kd transmembrane cell surface receptor that is a member of the tyrosine kinase family. HER-2 has been shown to be amplified in human breast(5), ovarian(6) and other cancers(7).
- C-MYC (8q24.12-q24.13) The MYC oncogene maps to the 8q24 region(8) and is involved in cell cycle progression.

- ZNF217 gene is a candidate oncogene located on 20q13.2 region(9).
- Cyclin D is another cell cycle related gene located on 11q13 and is often involved in breast cancer(10).
- The p53 (17p13.1) probe maps to the 17p13.1 region on chromosome 17 containing the p53 gene. In some malignancies, a mutation of one p53 gene allele is accompanied by a deletion of other alleles and results in the absence of wild-type p53 protein. Monoallelic deletion of p53 (as determined by FISH) is common in many disorders. Loss of heterozygosity of p53 has been identified in many tumors. The ability to use FISH probes such as the LSI p53 (17p13.1) for interphase cytogenetics has provided new insights into chromosomal aberrations. This probe may be used to detect the deletion (not mutation) or amplification of the p53 locus(11).
- The RB gene located at 13q14 is frequently deleted in many cancers(12).

Specifically, we looked for genetic instability in few breast cancer cell lines and few normal individuals. However, further studies are necessary to evaluate this novel method on a larger population to demonstrate its usefulness in identifying individuals at risk for breast cancer development.

MATERIALS AND METHODS

Study Design

Method Development and Validation

- Alkaline Comet-FISH v/s Neutral Comet-FISH
- Comet-FISH on untreated cells v/s cells with pre-imposed DNA damage/irradiation

Applications

- Comet-FISH on Breast cancer and normal cell lines
- Comet-FISH on normal individuals
- Comet-FISH on Breast cancer patients

Cell lines

Breast cancer cell lines

- HTB22
- HTB132
- CRL2336

Normal cell lines

- GM1310B
- AG11134

Comet Assay

Alkaline Comet assay

Cell lines and peripheral blood leukocytes from apparently healthy human volunteers/patients were used in this study. The alkaline Comet assay was performed according to Dr. Ray Tice's protocol as described in the Comet assay Interest Group Web site. Conventional glass microscope slides were coated with 1% normal melting point agarose and dried over a slide warmer. When the slides were fully dried, 5 µl of peripheral blood was mixed with 75µl of 0.5% low melting agarose (per slide) at 37°C and spread on the precoated slide. The slides were placed briefly on ice to allow the agarose to set, and immediately irradiated as described in the appropriate section. The irradiated slides were immediately placed in ice-cold lysis buffer (2.5 M NaCl, 100mM EDTA, 10 mM Trizma base, 10% DMSO, 1% Triton-X) and cells lysed overnight. After lysis, the slides were placed in horizontal electrophoresis tanks filled with electrophoresis buffer (300 mM NaOH / 1mM EDTA, pH 13.0) and DNA in the cells allowed to unwind for 20 minutes. The electrophoresis was then turned on and the slides electrophoresed at 25 V, 300 mA for 20 minutes. After electrophoresis, the slides were neutralized in neutralization solution (0.4 M Tris HCl, pH 7.5; three times 5 minutes each) and dried on

a slide warmer. The dried slides were stained with ethidium bromide (2μg/ml) and scored under fluorescence microscope using TRITC filters.

Neutral Comet assay

Cell lines and peripheral blood leukocytes from apparently healthy human volunteers/patients were used in this study. The neutral Comet assay was performed according to protocol as described in the Comet assay Interest Group Web site. Conventional glass microscope slides were coated with 1% normal melting point agarose and dried over a slide warmer. When the slides were fully dried, 5 μl of peripheral blood was mixed with 75μl of 0.5% low melting agarose (per slide) at 37°C and spread on the precoated slide. The slides were placed briefly on ice to allow the agarose to set, and immediately irradiated as described in the appropriate section. The irradiated slides were immediately placed in ice-cold lysis buffer (2.5 M NaCl, 100mM EDTA, 10 mM Trizma base, 10% DMSO, 1% Triton-X; pH 8.0) and cells lysed overnight. The slides were then incubated with Proteinase-K (1mg/mL of lysis solution without DMSO and Triton-X) for 60 minutes at 37°C. After lysis, the slides were placed in horizontal electrophoresis tanks filled with electrophoresis buffer (1x TBE, pH 8.0) and DNA in the cells allowed to equilibrate for 20 minutes. The electrophoresis was then turned on and the slides electrophoresed at 25 V, for 20 minutes. The current was adjusted to approximately 35-40 mA by raising or lowering the buffer. The dried slides were stained with ethidium bromide (2μg/ml) and scored under fluorescence microscope using TRITC filters.

Fluorescence in-situ hybridisation (FISH)

The protocol recommended by the probe manufacturer (Vysis Inc. Ltd, USA) was used in this study. The detailed protocol can be obtained from the Vysis.com website.

Comet-FISH

Alkaline Comet-FISH

This method involves hybridisation of slides after alkaline Comet assay with FISH probes. Exposure to high alkali during the alkaline Comet assay denatures the DNA and hence no further denaturation step is necessary.

Neutral Comet-FISH

This method involves hybridisation of neutral Comet assay slides with FISH probes. The DNA on neutral Comet assay slides are not denatured and hence requires an additional denaturation step. We tried 2 different types of denaturation step:

- **Heat denaturation**: The dried slides were denatured with 70% formamide at 72°C for 2 minutes.

- **Chemical denaturation**: The dried slides were denatured with 0.03M NaOH for 2 minutes at room temperature(4).

Preimposed irradiation

All irradiation were delivered in a Gammacell 40 Exactor research irradiator (MDS Nordion, Ontario, Canada) with 137Cs source at a specific dose rate of 1.0 or 1.22 Gy/min. All irradiation were performed over ice to prevent immediate rejoining of strand breaks induced by gamma radiation. Preliminary studies showed that after irradiation, the FISH signals were fragmented and scattered all over the comet and hence this method was not used.

Preimposed DNA damage

It was difficult to obtain efficient FISH signals after treatment with MMS or similar DNA damaging agent, and hence was not used in this study.

FISH Probes

All FISH probes were obtained from Vysis Inc., USA.. The details of the probes are given in Table 3.

RESULTS

BASE LINE COMET ASSAY OBSERVATIONS ON BREAST CANCER AND NORMAL CELL LINES. (TABLES 1 AND 2)

Table 1. Alkaline Comet assay results from breast cancer and normal fibroblasts.

Cell lines	Base line DNA damage			DNA damage after pre-imposed 2 Gy radiation		
	% DNA	Tail Length	Olive Tail Moment	% DNA	Tail Length	Olive Tail Moment
GM1310B	11.9 ± 2.8	21.3 ± 5.2	2.1 ± 0.5	33.1 ± 7.3	37.1 ± 4.5	8.1± 1.1
AG11134	12.5 ± 3.1	24.1 ± 7.2	1.9 ± 0.5	35.6 ± 6.7	39.6 ± 6.1	7.1 ± 1.4
HTB22	18.3 ± 3.1	29.5 ± 5.3	3.0 ± 0.8	45.1 ± 8.2	48.1 ± 4.8	10.3 ± 2.1
HTB132	16.9 ± 5.2	28.8 ± 4.1	3.1 ± 1.0	42.7 ± 3.2	49.6 ± 6.2	9.8 ± 1.2
CRL2336	19.2 ± 5.7	30.1 ± 5.2	3.0 ± 0.9	48.1 ± 6.6	51.2 ± 7.6	10.3 ± 2.9

Alkaline Comet assay: overnight lysis, unwinding at pH > 13.0; electrophoresis at pH>13.0, 25 V, 300 mA for 20 minutes. All values are (mean of 3 independent experiment) ± (standard error of the mean).

Table 2. Neutral Comet assay results from breast cancer and normal fibroblasts.

Cell lines	Base line DNA damage			DNA damage after pre-imposed 10 Gy radiation		
	% DNA	Tail Length	Olive Tail Moment	% DNA	Tail Length	Olive Tail Moment
GM1310B	10.1 ± 2.3	20.9 ± 1.4	0.81 ± 0.1	30.7 ± 5.6	36.2 ± 4.2	10.13 ± 2.7
AG11134	14.4 ± 2.1	19.2 ± 1.7	0.94 ± 0.2	28.8 ± 6.3	38.9 ± 5.1	10.37 ± 2.9
HTB22	20.2 ± 4.2	31.7 ± 3.1	1.2 ± 0.4	38.1 ± 7.1	45.1 ± 6.1	15.24 ± 3.1
HTB132	19.8 ± 4.8	30.9 ± 4.7	1.8 ± 0.5	42.4 ± 6.4	45.4 ± 5.3	15.54 ± 4.2
CRL2336	21.7 ± 4.9	28.7 ± 5.2	1.5 ± 0.4	40.8 ± 7.8	43.5 ± 6.1	14.60 ± 3.9

Neutral Comet assay: overnight lysis, Proteinase-K, 1mg/mL, 30 minutes at 37°C; electrophoresis at pH 8.0, 25 V, 40 mA for 20 minutes. All values are (mean of 3 independent experiment) ± (standard error of the mean).

BASELINE FISH OBSERVATIONS (TABLES 3, 4, AND 5)

Table 3. List of probes used in this study.

Probes	Probe Length	Gene Loci	Reference Probes
HER2 (Spectrum Orange)	~190 kb	17q11.2-q12	17 Alpha centromeric probe (Spectrum Green)
ZNF217 (Spectrum Orange)	~320 kb	20q13.2	-
Cyclin D (Spectrum Orange)	~300 kb	11q13	-
p53 (Spectrum Orange)	~145 kb	17p13.1	-
c-myc (Spectrum Orange)	~120 kb	8q24.12-q24.13	-
Rb (Spectrum Orange)	~220 kb	13q14.3	-

All probes from Vysis Inc.

Table 4. Baseline FISH observations using different FISH probes.

Cell lines	Percentage of cells with 2 signals				
	ZNF217	Cyclin D	p53	c-myc	Rb
GM1310B	88	87	90	92	84
AG11134	87	89	86	89	87
HTB22	90*	89	85	89*	54#
HTB132	85*	87	92	91*	28#
CRL2336	83*	75	84	81*	49#

* no evidence suggestive of amplification; # deletion of Rb gene seen in these cell lines
Cells were cytospin, fixed with Carnoy's fixative, and FISH performed as per the probe manufacturer's recommendation. a total of 400 cells were scored from each sample.

Table 5. Baseline FISH observations using the HER-2 probes.

Cell lines	HER-2
GM1310B	92% with 2 signals, good hybridisation efficiency
AG11134	95% with 2 signals, good hybridisation efiiciency
HTB22	81% with 2 signals; 8% with more than 2 signals; good hybridisation efficiency
HTB132	26% with 2 signals; 60 % more than 2 signals; good hybridisation efficiency
CRL2336	21% with 2 signals; 70% with more than 2 signals; good hybridisation efficiency

Cells were cytospin, fixed with Carnoy's fixative, and FISH performed as per the probe manufacturer's recommendation. a total of 400 cells were scored from each sample.

VALIDATION OF COMET-FISH METHOD (TABLES 6, 7, 8 AND 9)

ZNF217 probe was selected for the validation studies because of its large size (~320 kb).

Table 6. Classification system for Comet-FISH

Distribution of FISH signals in the comet	Interpretation
FISH signals in the Head	Stable genetic loci
Signals both in head and tail	labile genetic loci
FISH signals in the Tail	Unstable genetic loci

Table 7. Fluorescence in situ hybridisation with ZNF217 probe following alkaline Comet assay

	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	51	14	2	33
AG11134	45	7	3	45
HTB22	32	18	7	43
HTB132	37	19	5	39
CRL2336	40	9	9	42

The slides were hybridised with FISH probes following Alkaline Comet assay. Alkaline treatment at pH>13 would denature the DNA and help in probe hybridisation

Table 8. Florescence in situ hybridisation with ZNF217 probe on Neutral Comet assay slides denatured with 70% formamide at 72°C.

	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	63	11	5	21
AG11134	61	6	7	26
HTB22	59	2	10	29
HTB132	61	5	9	25
CRL2336	55	2	15	28

The slides were hybridised with FISH probes following Alkaline Comet assay. The alkaline treatment at pH>13 would denature the DNA and help in probe hybridisation

Table 9. Florescence in situ hybridisation with ZNF217 probe on Neutral Comet assay slides denatured with 0.03M NaOH, °C for 2.5 minutes, neutralised in 0.4M Tris (5 min) and TBE (2 min).

	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	73	9	5	13
AG11134	75	12	5	8
HTB22	71	9	5	15
HTB132	76	7	8	9
CRL2336	73	10	6	11

GENETIC INSTABILITY IN BREAST CANCER CELL LINES (TABLES 10-14)

Table 10. Genetic Instability at HER-2 loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	81	1	1	17
AG11134	87	2	0	11
HTB22	56	11	10	23
HTB132	19	20	43	18
CRL2336	14	18	50	18

Table 11. Genetic Instability at p53 loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	81	4	0	15
AG11134	75	3	2	20
HTB22	51	19	14	16
HTB132	45	16	29	10
CRL2336	37	21	28	14

Table 12. Genetic Instability at cyclin-D loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	79	1	3	17
AG11134	85	3	0	12
HTB22	81	1	5	13
HTB132	77	5	2	16
CRL2336	71	2	1	26

Table 13. Genetic Instability at c-myc loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	84	3	5	8
AG11134	78	7	3	12
HTB22	72	11	7	10
HTB132	76	9	4	11
CRL2336	71	2	7	20

Table 14. Genetic Instability at RB loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	75	5	12	8
AG11134	66	5	17	12
HTB22	13	12	14	61
HTB132	34	11	21	34
CRL2336	16	7	17	60

RESULTS OF GENETIC INSTABILITY IN APPARENTLY NORMAL INDIVIDUALS (TABLE 15)

Table 15. Genetic Instability in apparently normal individuals.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
HER-2				
A	86	3	1	10
B	81	2	0	17
P53				
A	89	5	1	5
B	83	7	1	9
ZNF217				
A	88	5	2	5
B	88	5	0	7
c-myc				
A	79	8	2	11
B	80	4	1	15
Cyclin-D				
A	83	8	0	9
B	71	11	1	17
Rb				
A	92	0	0	8
B	93	0	0	7

KEY RESEARCH ACCOMPLISHMENTS

- **Development and validation of a novel Comet-FISH technology for detecting genetic instability in breast cancers.**
- **Using the above method, we have shown that some breast cancer cells lines show genetic instability at HER-2 and p53 gene loci.**
- **This is a novel concept for detecting genetic instability at predefined genetic loci at the level of single cells.**

REPORTABLE OUTCOMES

- **We have developed and validated a new single cell diagnostic method.**
- **Has potential uses as a screening and epidemiological tool.**
- **Manuscript is under preparation and will be submitted to 'Cancer Research'.**

CONCLUSIONS

We have developed and validated a novel Comet-FISH method to detect breast cancer specific genetic instability. Of the three technical modifications tested, the combination of neutral Comet assay plus denaturation with 0.03M NaOH worked well in terms of good FISH signal intensity and specificity.

By applying this technique we further showed that some breast cancer cell lines exhibit instability at Her-2 and p53 loci. This method was further evaluated on apparently normal persons, who did not show genetic instability in any loci tested.

Further studies are necessary to evaluate this novel method on a larger population to demonstrate its efficiency in identifying persons at risk for breast cancer development.

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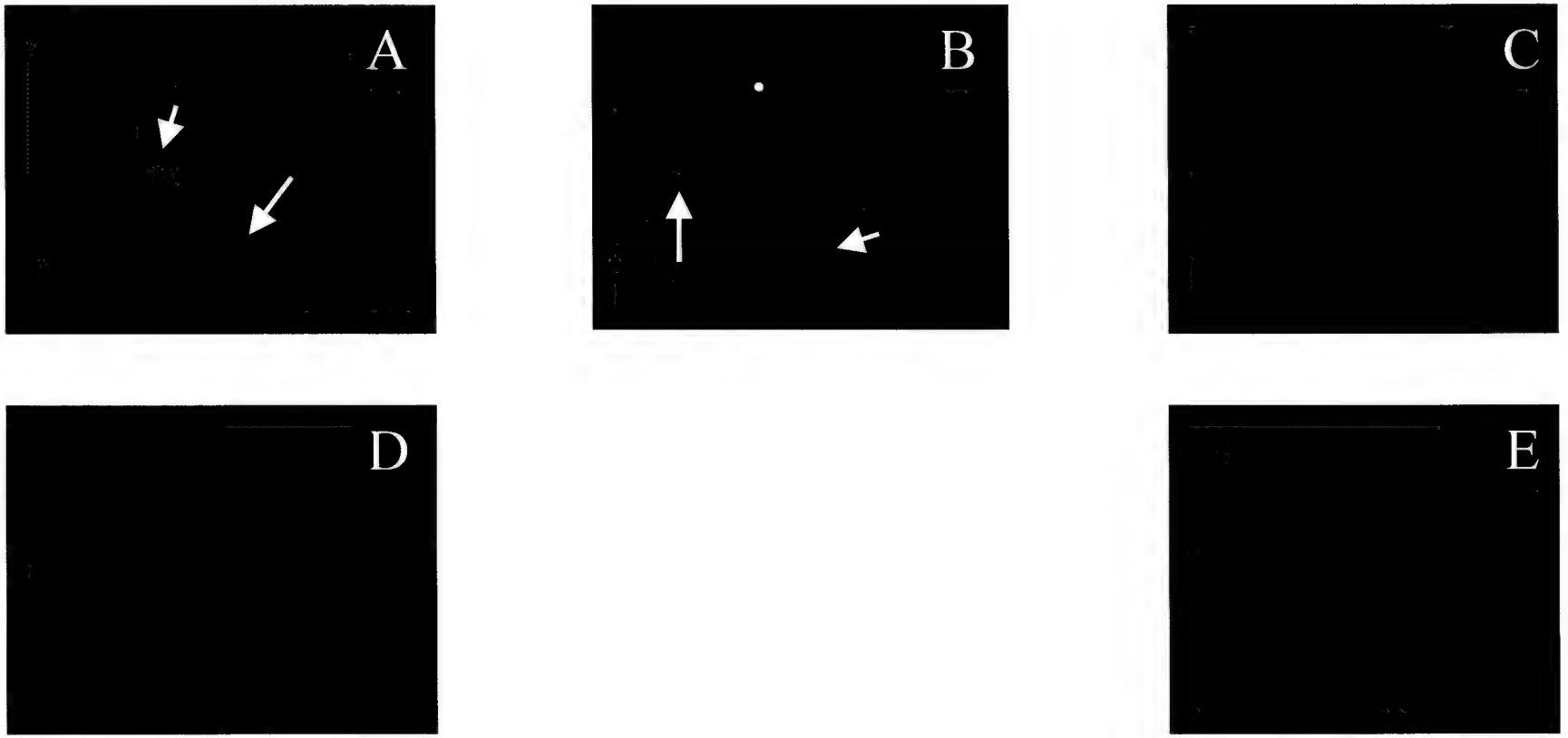
Representative photographs of Comet-FISH assay



APPENDIX-1

Figure legends

Representative photographs from Comet-FISH assay on breast cancer cell lines using various probes are shown in this figure. All images were taken using 60x oil immersion objective and suitably enlarged and color coded on computer, using Adobe Photoshop . (A) HER-2: note the FISH signals are in the tail. Most cells show more than 2 signals indicating amplification. (B and C) p53: Signals are seen in the head as well as in the tail. Few cells with deletion of single copy of p53 gene are also seen. (D) Rb: Most signals are in the head. Many cells show monoallelic deletion of Rb gene. (E) Cyclin D: Most signals are in the head of the comet.

Appendix-2

Synopsis of the poster presented at the 'Era of Hope' meeting at Orlando, 2002

Abstract

Certain women appear to have an increased risk of breast cancer. As compared with the general population, the first degree female relatives of breast cancer patients have been reported to have a 2- to 3-fold increase in breast cancer risk, which could be due to shared genetic and/or environmental components, and suggest a heterogeneity of population at risk. We hypothesize that persons prone for genetic instability at certain breast cancer associated gene locus are more prone to develop this cancer. We developed a novel 'Comet-FISH' technique to identify people who show genetic instability at these loci, and hence may be prone for developing breast cancer. By combining Comet assay and FISH techniques we can, not only identify cells with genetic instability, but also identify regions within the genome that are preferentially prone for instability. We applied this novel technique to look at genetic instability in predefined loci in breast cancer and normal cell lines, with and without pre-imposed radiation.

Hypothesis

- **It would be technically feasible to combine the two single cell assays, Comet assay and FISH.**
- **Comet-FISH can be used to examine genetic instability at specific genetic loci.**
- **Breast cancer cell lines show genetic instability at breast cancer associated gene loci.**
- **Persons exhibiting genetic instability at these loci are prone to develop breast cancers.**

Specific Aims

- **Develop and validate the Comet-FISH technology.**
- **To examine genetic instability in breast cancer/normal cell lines at pre-defined genetic loci.**
- **To examine genetic instability in apparently normal persons at these pre-defined genetic loci.**
- **To examine genetic instability in relatives of breast cancer patients, at these pre-defined genetic loci.**

Study Design

Method Development and Validation

- **Alkaline Comet-FISH v/s Neutral Comet-FISH**
- **Comet-FISH on untreated cells v/s cells with pre-imposed DNA damage/irradiation**

Applications

- **Comet-FISH on Breast cancer and normal cell lines**
- **Comet-FISH on normal individuals**
- **Comet-FISH on Breast cancer patients**

Methods

Alkaline Comet-FISH

This method involves hybridisation of slides after alkaline Comet assay with FISH probes. Exposure to high alkali during the alkaline Comet assay denatures the DNA and hence no further denaturation step is necessary.

Neutral Comet-FISH

This method involves hybridisation of neutral Comet assay slides with FISH probes. The DNA on neutral Comet assay slides are not denatured and hence requires an additional denaturation step. We tried 2 different types of denaturation step:

Heat denaturation: The dried slides were denatured with 70% formamide at 72°C for 2 minutes.

Chemical denaturation: The dried slides were denatured with 0.03M NaOH for 2 minutes at room temperature.

Classification System for Comet-FISH

Classification system for scoring Comet-FISH

Distribution of FISH signals in the comet	Interpretation
FISH signals in the Head	Stable genetic loci
Signals both in head and tail	labile genetic loci
FISH signals in the Tail	Unstable genetic loci

Neutral v/s Alkaline Comet-FISH

Alkaline Comet-FISH	Neutral Comet-FISH (Heat Denaturation)	Neutral Comet-FISH (0.03M NaOH Denaturation)
Many Cells with no signals	Many Cells with no signals	Efficient FISH hybridization

Therefore, neutral Comet-FISH followed by chemical denaturation was used in this study

Representative Photograph of Comet-FISH with HER-2 Probe

Computer generated Pseudo-colors

Representative Photograph of Comet-FISH with Rb Probe

Computer generated Pseudo-colors

Representative Photograph of Comet-FISH with p53 Probe

Computer generated Pseudo-colors

Genetic Instability at HER-2 in Breast Cancer Cell Lines

Cell lines	Percent cells with signals predominantly in the head	Percent cells with signals both in tail and head	Percent cells with signals predominantly in the tail	Percent cells with no FISH signals
GM1310B	81	1	1	17
AG11134	87	2	0	11
HTB22	56	11	10	23
HTB132	19	20	43	18
CRL2336	14	18	50	18

Genetic Instability at p53 in Breast Cancer Cell Lines

Cell lines	Percent cells with signals predominantly in the head	Percent cells with signals both in tail and head	Percent cells with signals predominantly in the tail	Percent cells with no FISH signals
GM1310B	81	4	0	15
AG11134	75	3	2	20
HTB22	51	19	14	16
HTB132	45	16	29	10
CRL2336	37	21	28	14

Genetic Instability at other loci in Breast Cancer Cell Lines

Other genetic loci tested did not show any genetic instability on Comet-FISH.

Genetic Instability in Two Apparently Normal Individuals

Did not show any genetic instability at any genetic loci tested.

Summary

Development and validation of a novel Comet-FISH technology for detecting genetic instability in breast cancers.

- **simple**
- **rapid**
- **specific for the genetic loci tested**

Certain breast cancer cells lines show genetic instability at HER-2 and p53 gene loci.

Novel concept for detecting genetic instability at predefined genetic loci at the level of single cells.

Future Direction

To evaluate this novel method on a larger population to demonstrate its efficiency in identifying persons at risk for breast cancer development.